



## Kinetic analysis of site-directed mutants of methionine synthase from *Candida albicans*

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### ABSTRACT

Fungal methionine synthase catalyzes the transfer of a methyl group from 5-methyl-tetrahydrofolate to homocysteine to create methionine. The enzyme, called Met6p in fungi, is required for the growth of the pathogen *Candida albicans*, and is consequently a reasonable target for antifungal drug design. In order to understand the mechanism of this class of enzyme, we created a three-dimensional model of the *C. albicans* enzyme based on the known structure of the homologous enzyme from *Arabidopsis thaliana*. A fusion protein was created and shown to have enzyme activity similar to the wild-type Met6p. Fusion proteins containing mutations at eight key sites were expressed and assayed in this background. The D614 carboxylate appears to ion pair with the amino group of homocysteine and is essential for activity. Similarly, D504 appears to bind to the polar edge of the folate and is also required for activity. Other groups tested have lesser roles in substrate binding and catalysis.

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Methionine synthases catalyze the transfer of a methyl group from the tertiary amino group of 5-methyl-tetrahydrofolate (5-methyl-THF) to the sulfhydryl group of L-homocysteine (Hcy) to synthesize L-methionine, and regenerating THF. There are two types of methionine synthase: (1) cobalamin-dependent methionine synthase and (2) cobalamin-independent methionine synthase. The former, encoded in *Escherichia coli* by the MetH gene, makes use of a cobalamin prosthetic group for methyl transfer. The cobalamin-independent form, encoded in *E. coli* by the MetE gene, performs direct methyl transfer [1]. The MetH gene homolog is seen in animals (including humans), eubacteria, and eukaryotes, while the MetE homolog is seen in plants, insects, fungi, archaea, and eubacteria, which are deficient in vitamin B12. In yeast, and other fungi, the gene encoding the B12-independent enzyme is referred to as MET6 and the corresponding protein is Met6p. Although both of these enzymes carry out the same overall reaction, there is no sequence similarity between the two [2]. The MetH gene product is a 140 kDa protein consisting of four distinct domains for binding Hcy, 5-methyl-THF, cobalamin, and S-adenosyl-methionine [3]. The MetE gene product is an 84 kDa protein; X-ray structures have been solved for the enzymes from *Arabidopsis thaliana* and *Thermatoga maritima* [4,5].

*Candida albicans* is one of the major opportunistic pathogenic species of *Candida* [6]. Since the methionine synthase in fungi is

structurally and mechanistically different from its human counterpart, it makes a suitable antifungal drug target. No X-ray structure of a fungal Met6p enzyme has yet been solved. Based on sequence alignment, the fungal and plant enzymes are about 50% identical, and amino acids in the active site are highly conserved. In order to help elucidate details of the mechanism of methionine synthase, we report here the cloning, expression, and kinetic analysis of site-directed active site mutants of cobalamin-independent methionine synthase from *C. albicans* as glutathione S-transferase (GST) fusion proteins.

### Materials and methods

**Materials.** Glutathione agarose beads and reduced glutathione were purchased from Sigma Chemical (St. Louis, MO). PteroylGlu<sub>3</sub> and (PbNO<sub>3</sub>)<sub>2</sub> for the synthesis of 5-methyl-THF were purchased from Schircks Laboratories (Jona, Switzerland) and Mallinckrodt, respectively. L-Homocysteine thiolactone for the synthesis of homocysteine was purchased from Sigma. All primers were obtained from Integrated DNA Technologies (Coralville, IA). pRS424, the yeast expression vector, was obtained from Dr. Arlen Johnson (The University of Texas at Austin).

**Cloning and expression of *C. albicans* methionine synthase as a GST fusion protein.** The pEGKT vector is used for galactose-inducible overexpression of GST fusion proteins in yeast [7]. We obtained it from Dr. Clarence Chan (The University of Texas at Austin), and used it, in part, to construct a plasmid expressing *C. albicans* methi-

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onine synthase as a GST fusion protein. We replaced the pEGKT *CYC1* promoter with the constitutive *Saccharomyces cerevisiae* methionine synthase promoter; mutagenesis removed the XhoI and SacI restriction sites, resulting in only one XhoI site and one SacI site flanking the *CYC1* promoter. The inducible promoter was removed using an XhoI/SacI double digest. The *ScMET6* promoter was PCR-amplified from the Yep24-*CaMET6* construct and then cloned into the XhoI and SacI sites on the pEGKT vector. Since the GAL1-10 upstream activation sequence (UAS) was not removed from the pEGKT vector, it repressed the *ScMET6* promoter. The newly constructed *ScMET6* promoter-GST fragment was removed from pEGKT using XhoI and XmaI restriction enzymes and ligated into the XhoI and XmaI restriction sites on the pRS424 vector. The *CaMET6* gene was PCR-amplified from the Yep24-*CaMET6* construct [8] using suitable primers and cloned into the XmaI restriction site on the pRS424 vector. This construct, pRS424-GSTCaMET6, was sequenced to ensure no mutations occurred during PCR and that the *CaMET6* gene was in frame with the GST. pRS424-GSTCaMET6 was transformed into the *met6* disruptant strain SDY $\alpha$  [8] and transformants selected by their ability to grow in the absence of exogenous methionine. Transformants were grown to confluence in 100 ml YMD media supplemented with L-serine (375 mg/L), L-leucine (30 mg/L), and L-histidine (20 mg/L) at 30 °C while shaking. This culture was used to inoculate a 1000 ml media and grown at 30 °C while shaking until an OD<sub>600</sub> of 3–4 was reached. The cells were harvested by centrifuging at 5000g for 20 min at 4 °C.

**Purification of GST-CaMet6p.** The harvested cells were resuspended in 1× phosphate buffered saline (PBS; 2 ml/g wet weight of the pellet) and 1 ml of protease inhibitor cocktail solution (Roche Applied Science, Indianapolis, IN) was added. The cells were disrupted by passing four times through a 20 K French press cell operating at 1000 psi. The disrupted cells were centrifuged at 20,000g for 1 h at 4 °C in a Beckman ultracentrifuge to obtain the cell lysate. The cell lysate was filtered through a 0.2  $\mu$ m filter to remove any floating material, and loaded onto a 2.5 ml glutathione agarose column. The flow through was reloaded twice onto the column to decrease protein loss in the flow through. The column was washed with five column volumes of 1× PBS, followed by elution of the fusion protein, GST-CaMet6p (102 kDa), using 1× PBS containing 10 mM reduced glutathione. The eluate fractions containing protein were pooled and dialyzed overnight at 4 °C against 50 mM Tris-Cl, 10 mM BME; pH 7.4. The purified protein was concentrated in an iCON concentrator (Thermo Scientific, Rockford, IL) by centrifuging at 6000 rpm at 4 °C, flash frozen in liquid nitrogen in 50  $\mu$ l aliquots, and stored at –80 °C until needed.

**Site-directed mutagenesis.** Site-directed mutagenesis was performed as per the protocol of the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) using KOD Hot Start DNA Polymerase (Novagen). Primers were designed to be complementary to the sense and antisense strands of the template (pRS424-GSTCaMET6), and the desired mutation was in the middle of the primer. Briefly, pRS424-GSTCaMET6 was used as a template in a PCR using KOD Hot Start DNA Polymerase and the complementary primers. The PCR product was incubated with the restriction enzyme DpnI at 37 °C for 2 h to digest the parental methylated DNA. DpnI-digested DNA was transformed into competent *E. coli* cells and transformants were obtained on LB plates containing ampicillin 100  $\mu$ g/ml. The plasmid containing the desired mutation was obtained from an overnight culture of the transformants using QIAprep Spin Miniprep kit from Qiagen and transformed into the *met6* disruptant strain SDY $\alpha$  [8]. The transformed cells were plated on YMD plates plus or minus methionine. Twelve point mutants were made: S448A, E620Q, E620S, D504A, D504N, D504S, D614S, W576F, W576Y, H128N, R530A and Y527F. These mutations were identified using a model of CaMET6, which was made by threading

its sequence onto the structure of the cobalamin-independent methionine synthase from *A. thaliana* (PDB code 1U1H) [8] using the online SCWRL server at the Joint Center for Structural Genomics (<http://www.jcsg.org/prod/scripts/home.html>). Hydrogen atoms were added to the CaMet6p model and it was energy minimized using ICM [9].

**Enzyme assay.** A modification of the methionine synthase assay described by Drummond et al. [10] was employed to determine enzyme activity. THF cofactors can exist in a variety of poly-glutamated states; we showed the fungal Met6p enzymes require at least two glutamates for activity [12], but we routinely synthesize 5-methyl-THF with three glutamates. (6R,S)-5-Methyl-THFGlu<sub>3</sub>, was synthesized from PteGlu<sub>3</sub> using a modified procedure of Yeo and Wagner [8,11]. For convenience, we will refer to this substrate simply as 5-methyl-THF. The substrate L-Hcy was prepared by alkaline hydrolysis of L-homocysteine thiolactone [10]. A standard reaction was performed in 400  $\mu$ l using 9  $\mu$ g GST-CaMet6p, 400  $\mu$ M L-Hcy and 500  $\mu$ M 5-methyl-THF. The reaction mixture, which also consisted of 50 mM potassium phosphate (pH 7.2), 50 mM Tris-Cl (pH 7.2), 100  $\mu$ M MgSO<sub>4</sub>, and 10 mM dithiothreitol, was pre-incubated at 37 °C for 10 min. After the addition of the enzyme, the reaction was run for 10 min at 37 °C. One hundred microliters of 5 N HCl/60% formic acid was added to stop the reaction, followed by incubation at 80 °C for 10 min to convert THF to its derivative, methenyl-THF. The reaction mixture was cooled to room temperature and precipitated protein pelleted by centrifugation. Methenyl-THF was quantified spectroscopically by measuring absorbance at 350 nm, where it has an extinction coefficient of 26,500/M/cm. The *K<sub>m</sub>* of 5-methyl-THF was determined by assaying at varying concentrations of 5-methyl-THF (0–500  $\mu$ M) keeping the L-Hcy concentration at a near saturating level of 400  $\mu$ M. Similarly, the *K<sub>m</sub>* of L-Hcy was determined by assaying at varying concentrations of L-Hcy (0–200  $\mu$ M) keeping the 5-methyl-THF concentration at 500  $\mu$ M.

## Results and discussion

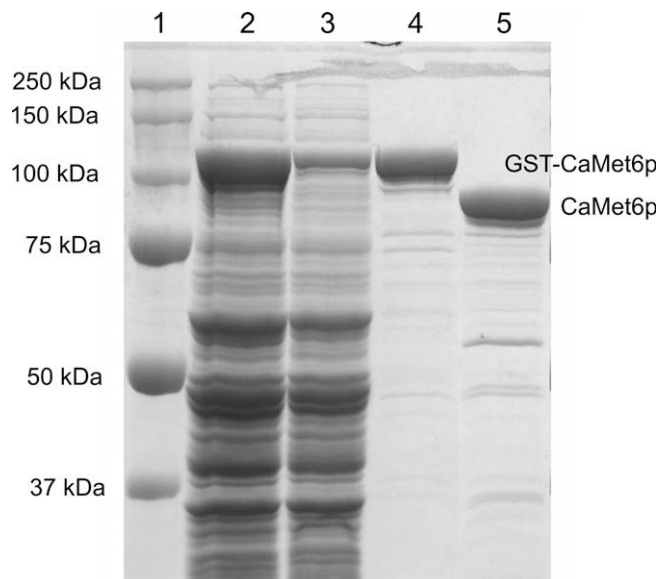
### Purification of GST-CaMet6p

Candida methionine synthase was expressed from the pRS424 shuttle vector as a fusion protein with GST at its N-terminus; we will refer to this fusion protein as GST-CaMet6p (102 kDa). Previous attempts to express point mutants using the Yep-*CaMET6* construct, were unsuccessful. Western blots suggested the proteins were unstable and rapidly degraded. The stability, and solubility, of some proteins is known to increase when expressed as a fusion protein with GST, which led to the notion of expressing CaMet6p as a fusion. The SDY $\alpha$  strain used to express the fusion protein has a disrupted *met6* gene and does not exhibit any methionine synthase activity of its own.

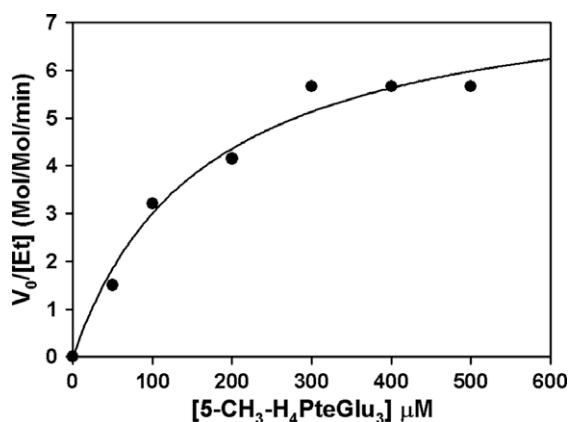
GST-CaMet6p was purified on a glutathione agarose column that exhibits high affinity for the GST. Fig. 1 shows an 8% SDS–polyacrylamide gel electrophoresis of the fractions from the purification. Molecular weight standards are in lane 1 with labels on the left. Lane 2 is the cell extract, and lane 4 is the eluate from the GST affinity column. The major band runs at the anticipated molecular mass of 112 kDa; that is, 26 kDa for GST plus 86 kDa for Met6p. Lane 5 shows purified CaMet6p, 86 kDa. One liter of SDY $\alpha$ /pRS424-GSTCaMET6 cells gave 4 mg of GST-CaMet6p. This yield is only slightly lower than that seen for the wild-type protein [8].

### Kinetic analysis

A standard enzyme assay [12] was performed on the GST-CaMet6p fusion protein. Fig. 2 shows the steady state kinetic data



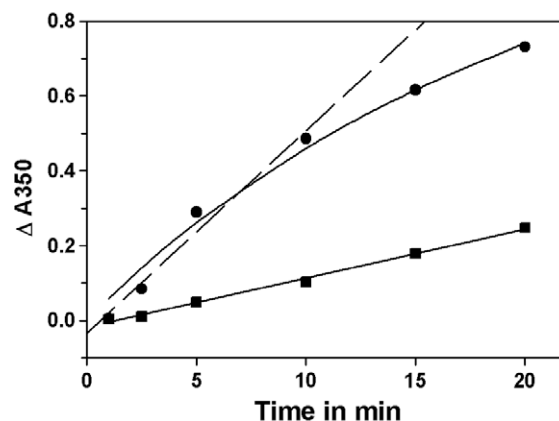
**Fig. 1.** SDS-PAGE of GST-CaMet6p purification. An 8% SDS-polyacrylamide gel was run using protein markers (lane 1), 10  $\mu$ l crude cell extract (lane 2), 10  $\mu$ l column flow through (lane 3), the pooled eluate, 2  $\mu$ g GST-CaMet6p (lane 4). CaMet6p (2  $\mu$ g) was run on lane 5 as a comparison. The molecular weights of the protein markers are shown on the left.



**Fig. 2.** Steady state kinetic curve for GST-CaMet6p. The concentration of 5-methyl-THF is varied while the concentration of Hcy is saturated at 400  $\mu$ M.

for CaMet6p, as a function of 5-methyl-tetrahydrofolate, in the presence of effectively saturating concentration of Hcy, 0.4 mM. Kinetic parameters were computed from a hyperbolic fitting of the data. A parallel plot varying Hcy in the presence of saturating 5-methyl-THF was also made (data not shown). The  $K_m$  values of 5-methyl-THF and Hcy are 165  $\mu$ M and 29  $\mu$ M, respectively. These  $K_m$  values are only slightly higher than the 129  $\mu$ M and 14  $\mu$ M values observed for 5-methyl-THF and Hcy, respectively, with the purified CaMet6p [8]. The turnover number,  $k_{cat}$ , for GST-CaMet6p was observed to be around 8/min, slightly lower than that for CaMet6p (25/min).

Many of the site-directed mutant proteins had activity levels too low to accurately assess steady state kinetic parameters. Instead, we compared the overall rate of the mutants with the GST-CaMet6p protein, at fixed substrate concentrations with 400  $\mu$ M Hcy and 500  $\mu$ M 5-methyl-THF. Fig. 3 shows one example. The time course for the wild-type GST-CaMet6p is shown as circles, and can be fit to a hyperbola over 20 min as shown by the solid line. It is clear that substrate is being exhausted. The initial rate,



**Fig. 3.** Time Course assay of GST-CaMet6p versus GSTCaMet6pW576F. A standard enzyme assay was performed and  $\Delta A_{350}$ , proportional to product formation, is plotted against time in minutes. Data for the wild-type is closed circles and that for the mutant GSTCaMet6pW576F is squares.

over about 10 min, is shown as a dashed line. The less active W576F mutant exhibits linear production over 20 min, as indicated by its least squares fit. The ratio of the initial slopes of wild-type to the mutant is 4.1, and this is taken as their relative activities.

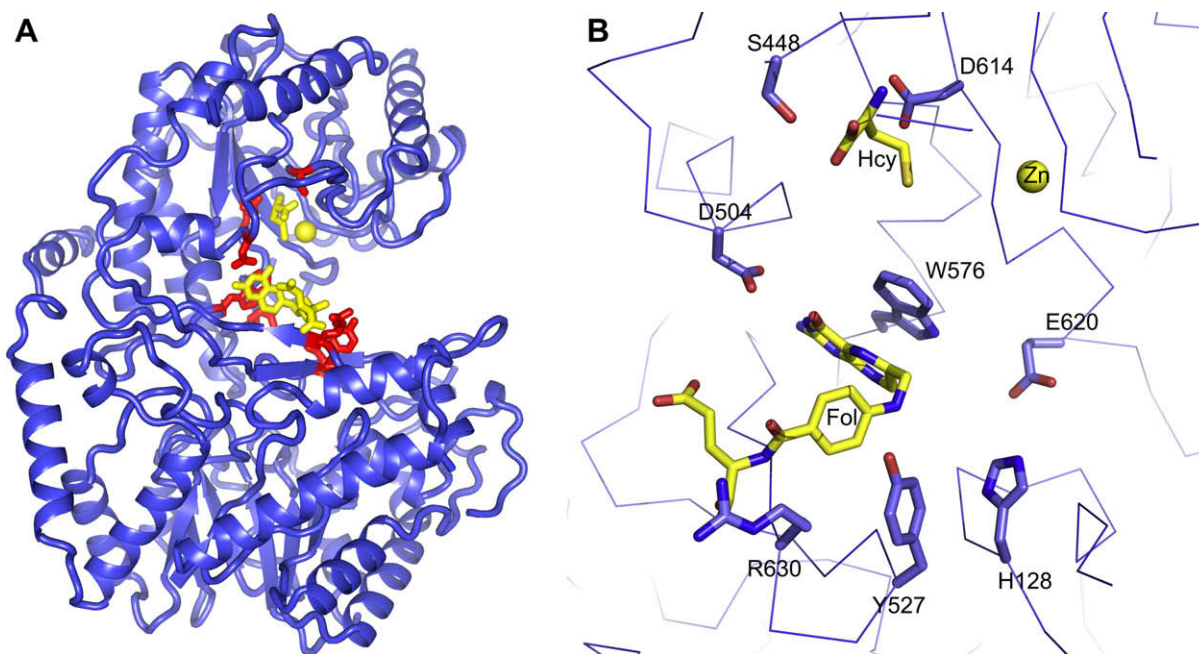
#### Site-directed mutagenesis

Site-directed mutants of *C. albicans* methionine synthase were made to assess the roles of some of the active site amino acid residues in catalysis. The amino acid sequence of *C. albicans* methionine synthase was deduced from an open reading frame analysis of the genome, which identified the Met6p protein [12]. This sequence was aligned with that of *A. thaliana* and a model of CaMet6p was made by threading the *C. albicans* sequence unto the known *A. thaliana* structure and then carrying out energy refinements [13] to remove minor side chain clashes. Key active site residues were deduced by examining the structure, and putative interaction with substrates, as seen bound to the *A. thaliana* structure [4]. Fig. 4A shows the model and Fig. 4B shows a detail of the active site. Eight residues were chosen to be mutated; these are labeled in Fig. 4B. All of the residues are plausible candidates for catalytic roles based on their positions, and all are conserved across the family of cobalamin-independent methionine synthases. We used the BLAST alignment server at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) to compare 10 fungal and bacterial sequences; this showed all eight residues were invariant (data not shown). These point mutants were expressed in the *met6* disruptant strain, SDY $\alpha$ , and purified in exactly the same way as the wild-type fusion protein, GST-CaMet6p. Table 1 shows whether these mutant proteins were able to rescue the methionine auxotrophy of the SDY $\alpha$  strain, when grown in YMD media in the absence or presence of methionine. A mutant with negligible methionine synthase activity can only grow in the presence of exogenous Met. Once expressed, and purified, a standard enzyme assay was performed using both the mutant and wild-type fusion protein, and their specific activities were compared.

#### Analysis of the mutant enzymes

In the native enzyme, S448 and D614, appear to form hydrogen bonds to Hcy (Fig. 4B). Our model suggests the S448 hydroxyl might donate a hydrogen bond to the carboxylate of Hcy. The model also suggests that the carboxylate side chain of D614 forms an ion pair with the Hcy amine group; the co-crystal structure of *A. thaliana* MetE protein with Met shows such an interaction between





**Fig. 4.** A model of the active site of *Candida albicans* methionine synthase. (A) The backbone is shown in blue. The position of the active site Zn, Hcy, and folate are shown in yellow. Residues subject to mutagenesis are indicated in red. (B) Close up of the active site with key groups labeled; figures were made using PyMol [18].

**Table 1**

Growth of the SDY $\alpha$  strain expressing GST-CaMet6p mutants. Each mutant name is followed by two columns. The first indicates if the mutant enzyme can support growth on YMD media without supplemented methionine; all mutants could grow on plates supplemented with methionine. The second column indicates the % activity of the purified mutant enzyme with respect to wild-type GST-CaMet6p.

Mutant	Growth on - Met media	GST-CaMet6p activity (%)	Mutant	Growth on - Met media	GST-CaMet6p activity (%)
S448A	Yes	67	D614S	No	11
E620Q	Yes	100	D614A	No	<2
E620S	Yes	100	D614N	No	<2
D504A	No	4	W576F	Yes	30
D504N	No	11	W576Y	No	12
D504S	No	7	R530A	Yes	33
H128N	No	19	Y527F	No	67

the homologous Asp 605 and Met [4]. To measure their contributions to substrate binding, S448 was mutated to alanine (S448A). D614 was converted to alanine (D614A), serine (D614S), and asparagine (D614N). The S448A protein exhibited 67% of wild-type activity, indicating that the Ser side chain is not crucial for substrate binding. The relatively high enzyme activity is consistent with the ability of this mutant protein to rescue the methionine auxotrophy of the SDY $\alpha$  strain, as shown in Table 1.

In contrast, D614 appears to play a much more important role in Hcy binding. None of the D614A, D614S, and D614N mutants could sustain growth on (-Met) media (Table 1). Only the D614S protein gave any measureable enzyme activity. Apparently the ion pair between the D614 side chain and Hcy ammonium group is essential for substrate recognition and binding. The side chain of the D614N mutant could, in principle, receive a hydrogen bond from the ammonium group, but this interaction is apparently not strong enough to assure effective substrate binding. It is generally accepted that ionic interactions can strongly influence substrate diffusion toward the active site and facilitate binding [14]; it may be that the charged D614 side chain plays this role in attracting, orienting, and binding the Hcy substrate.

D504 appears to make specific interactions with the polar edge of the folate ring; in particular it receives hydrogen bonds from N3 and the exocyclic amine of C2. Binding and orienting folate using a protein carboxylate is a common feature of folate dependent enzymes [15,16]. Mutation of D504 to alanine, serine, or asparagine was found to eliminate measurable enzyme activity. This suggests that D504 plays a crucial role in binding and aligning the folate substrate.

E620 is also close to the folate ring; its homolog in the enzyme from *T. maritima* was seen crystallographically to interact with a folate analog, albeit in a conformation considered to be non-productive [5]. To assess its role, we mutated E620 to glutamine and serine. Neither mutation had an effect on enzyme activity, confirming that it does not play a major role in productive folate binding.

W576 stacks with the pyrimidine ring of the folate cofactor; this stacking has been reported in the X-ray structure of the *A. thaliana* [4] and *T. maritima* enzymes [5]. To test whether W576 of *Candida* methionine synthase behaves similarly, it was mutated to a phenylalanine and tyrosine. The W576F mutant and the W576Y mutants were 3-fold and 8-fold less active, respectively, compared to the wild-type fusion protein. It is obvious from these data that W576 interacts with the pterin ring of 5-methyl-THF, but that other aromatic platforms can also stack with the co-substrate. It may be that the indole ring of Trp is superior at orienting the folate N5 atom carrying the methyl group in a direction enabling methyl transfer to Hcy.

Methyl transfer from 5-methyl-THF is a conceptually difficult reaction given that tertiary amines are such poor methyl donors [1]. It has been speculated that the transfer would be facilitated by protonation at N5. Histidine 128 is adjacent to 5-methyl-THF in our model; we wanted to test the notion that it might serve as a general acid to protonate the folate N5. Alternatively the histidine might play some lesser role in binding or orienting the folate. When mutated to asparagine, the protein was 5-fold less active than the wild-type fusion protein. Asn is still able to make polar contacts similar to those of His, but it cannot act as an acid. Our observation is consistent with a relatively minor role in enzyme catalysis and appears to rule out a role as a mechanistic acid.

R530 and Y527 are in position to interact with the poly-glutamate tail of the folate cofactor. To assess their importance they were mutated to alanine and phenylalanine, respectively. Removal of the R530 guanidinium in R530A reduced overall activity to 33% while removal of the Y527 hydroxyl in Y527F reduced overall activity to about 67%. These results confirm that the residues play minor, but real, roles in binding the folate tail.

We have shown that fungal methionine synthase is a potential target for antifungal drug design [17]. The studies reported here help increase our understanding of the active site of the enzyme and may help in the rational design of inhibitors. For example, Table 1 shows the W576F, but not the W576Y, has sufficient activity to support growth on a (-Met) media. That is 30% but not 12% of Met6p activity will support growth. This suggests that even modest inhibitors of fungal Met6p enzymes could seriously retard fungal growth. This study indicates it is profitable to consider both the folate and homocysteine binding sites in the design of enzyme inhibitors. The large hydrophobic folate binding area near W567 will need to be exploited to assure reasonable binding affinity. Pendant groups that bind the homocysteine site, particularly interacting with D614, could provide drug specificity reducing undesired inhibition of other folate dependent enzymes.

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